MINISTRY OF HEALTH

GUIDELINES FOR CERTIFICATION OF ONCHOCERCIASIS ELIMINATION IN UGANDA

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ACRONYMS

APOC	African Programme for Onchocerciasis Control
BNI	Bernhard Nocht Institute for Tropical Medicine, Hamburg
CDC	Communicable Disease Control
CDTI	Community Directed Treatment with Ivermectin
CMFL	Community Microfilaria Load
DNA	Deoxyribonucleic Acid
DNA	Deoxyribonucleic acid
DTT	Dithiorythritol
ELISA	Enzyme Linked Immunosorbent Assay
GOU	Government of Uganda
GTZ	German Technical Cooperation
HCL	Hydrochloric acid
HSSIP	Health Sector Strategic Investment Plan
IACO	International American conference on Onchocerciasis
ICT	International Certification Team
MDP	Mectizan Donation Programme
MOH	Ministry of Health
NCC	National Certification Committee
NMS	National Medical Stores
NOCP	National Onchocerciasis Control Programme
OD	Optical Density
OEPA	Onchocerciasis Elimination Programme of the Americas
PCR	Polymerase Chain Reaction
PES	Post Endemic Surveillance
PTS	Post Treatment Surveillance
RBF	River Blindness Foundation
RTI	Research Triangle International
SCI	Schistosomiasis Control Initiative
SDS	Sodium Dodecyl Suphate
SS	Sightsavers
SSPE	Saline Sodium Phosphate
STP	Seasonal Transmission Potential
UOECC	Uganda Onchocerciasis Elimination Certification Committee
UOEEAC	Uganda Onchocerciasis Elimination Expert Advisory Committee
USAID	United States Agency for International Development
VHT	Village Health Team
WHO	World Health Organization

PREFACE

Human Onchocerciasis (river blindness) is a public health problem in several Sub-Saharan African countries including Uganda. In Uganda the disease is endemic in 35 districts with estimated 1.4 million people affected and more than two million at risk of being infected by the disease. The disease has been categorized as one of the underlying causes of poverty amongst the communities where it is prevalent. Guidelines on certification of onchocerciasis elimination in Uganda have been developed based on the experiences gained during the elimination of diseases like the small pox, poliomyelitis and dracunculiasis. The document sets forth the benchmarks to be used to ascertain if indeed onchocerciasis transmission has been interrupted and hence cessation of intervention in any focus. It also sets forth the core activities to be accomplished during interruption of transmission as part of post treatment surveillance, a process for certification of the country as well as the immediate post certification interventions to check recrudescence or re-invasion by the disease vectors. Despite the challenges facing the health ministry, it is envisaged that the goal of onchocerciasis elimination in Uganda will be achieved in the near future. The document is meant to be used by all stakeholders involved in the control and elimination of the disease at all levels of the health care delivery system as well as the national certification committee who are mandated with this responsibility.

The document has been developed in partnership with all stakeholders involved in the control and elimination of onchocerciasis in Uganda. The health ministry remains committed to the policy of onchocerciasis elimination in Uganda and will put in place an effective surveillance system to monitor the disease situation during the post-elimination period in all formerly endemic foci and districts in Uganda.

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The development of this document arose out of a felt need to have a simple standard tool for verifying claims of local interruption of onchocerciasis transmission in any focus and which would form the basis of cessation of intervention and pave way for certification of elimination. Its development was based on WHO guidelines on certification of human onchocerciasis and experiences acquired from the certification process of dracunculiasis in Uganda.

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1.0 Introduction

Human onchocerciasis is one of the diseases targeted for elimination in Uganda. The disease is endemic in 35 districts with estimated 1.4 million people affected and more than two million at risk of infection. The disease has been categorized as one of the underlying causes of poverty amongst the communities where it is prevalent (see Appendix 2).

In an effort to scale up national response for the disease elimination in Uganda, the Government of Uganda launched onchocerciasis elimination campaign in January 2007. The policy aims to eliminate onchocerciasis through semi-annual community directed treatment with ivermectin combined with vector control/elimination (WHO, 1996). The policy shift was a result of observations both here and in OEPA that annual treatment alone not only failed to eliminate infections but prolonged period of interventions in order to achieve interruption of disease transmission (Ndyomugyenyi, *et.al.*, 2004; Katabarwa, *et. al.*, 2008; Garms, *et.al.*, 2009; Winnen, *et al.*, 2002; Cupp & Cupp, 2005). Currently fourteen districts in six foci (Budongo, Bwindi, Kashoya-Kitomi, Mt Elgon, Wambabya-Rwamarongo, Wadelai) are implementing these strategies where tremendous progress has been made in interrupting disease transmission. There is however concern whether to halt or to continue endlessly with these interventions in these foci where transmission has been interrupted. Against this background, the MOH decided to develop guidelines based on epidemiological and entomological indicators that will be used to verify transmission status needed before any foci can be certified free of onchocerciasis, and necessitating the halting of interventions in any foci in Uganda. The guidelines also provide a framework of post eliminations interventions to be implemented to ensure that any foci which have been freed from onchocerciasis are not re-infested by the disease vectors.

1.1 Justifications for the guidelines

The rationale for the development of guidelines for certification of elimination stems from the need to have a single standard protocol to document, monitor, and evaluate the process of implementing the new policy as part of national preparedness. These guidelines provide the mechanism to:-

a) Ensure credibility for future claims that onchocerciasis has been eliminated from a defined focus.
b) Judging the success of the national programme in elimination of onchocerciasis.

2.0 ONCHOCERCIASIS CONTROL AND ELIMINATION INTERVENTIONS

Two strategies are currently employed for onchocerciasis control among the affected communities in Uganda. These interventions are implemented in partnership with GOU, the development partners and the communities in the context of decentralization. The main thrust focuses on preventive chemotherapy and vector control/elimination as described below.

2.1 Preventive chemotherapy

In most parts of Africa where the vectors are widespread and disease foci are not isolated (usually where *S. damnosum s.l.* is the vector) treatment of the affected communities with ivermectin is the approach of choice (WHO, 1995, WHO/APOC, 2009). In Uganda efforts to control the disease were initiated in 1950s by the Vector Control Division of MOH by dosing breeding sites with DDT.

Although these operations were successful in the Victoria Nile focus, it came to a halt in 1977 due to civil unrest (Barnley, 1975; McCrae, 1978; Prentice 1974). Onchocerciasis control in Uganda was resumed in the early 1990's using annual community based ivermectin (Mectizan) distribution. In 1996 the community directed treatment with ivermectin was introduced in partnership with development partners and community involvement for sustainability of the intervention (WHO, 1996). The intervention was successful in reducing the prevalence of microfilariae from 70% in 1993 to 7% but without interruption of transmission in most of the endemic foci in the country (Ndyomugyenyi *et al.,* 2007; Garms *et. al.,* 2009). The challenge with this strategy is that a high level of coverage in terms of geographic, programmatic and therapeutic coverage must be attained and motivation of the community ivemectin distributors in order to sustain the intervention.

2.2 Vector control/elimination

In areas where onchocerciasis foci are relatively small and isolated and where *S. neavei* is the vector, the vector elimination intervention is preferred in combination with CDTI. Currently this strategy is being implemented in Uganda where onchocerciasis is predominantly transmitted by *S. neavei* and has been found to be very effective in controlling onchocerciasis (Walsh *et al.*, 1996, Nydyomugyenyi *et al.* 2004; Garms *et al.* 2009). Temephos (Abate) is the chemical used for dosing *S. neavei* breeding sites in Uganda and has been effective in eliminating the vector in Itwara focus in Kabarole/Kyenjojo districts and prospects are high to eliminate the vector in the Mpamba-Nkusi and Elgon foci (Lakwo, *et. al.*, 2006; Richards, *et al.,* 2009).

2.3 Onchocerciasis elimination

The goal of the onchocerciasis elimination strategy in Uganda is to eliminate new infections due to *Onchocerca volvulus* by the year 2015 in areas where elimination intervention are currently being implemented, followed by the rest of the country by 2020. To guide implementation process "Oncho flag" (Appendix 1) was developed showing different colour codes to indicate the various transmission status and hence interventions. The flag was adopted from the Onchocerciasis Elimination Programme of the Americas (OEPA) where feasibility of eliminating onchocerciasis was achieved (Sauerbrey, 2008; Lindblade *et al.* 2007; Cupp & Cupp, 2005). The whole country has been mapped and currently there are 15 districts implementing semi-annual treatment while 20 districts are implementing the annual treatment strategy. It is envisaged that between two categories of districts, interventions will be phased to include semi-annual distribution of ivermectin, to semi-annual distribution of ivermectin with vector elimination and semi-annual distribution of ivermectin with targeted vector control. Therefore the "Oncho flag" will be reviewed regularly and updated based on the progress of elimination campaign.

TABLE 1: UGANDA ONCHOCERCIASIS ELIMINATION FLAG.

No.	Colour zone	Meaning of colour zone
1	Dark green	Onchocerciasis eliminated (3 year PTS completed)
2	Light green	Onchocerciasis transmission interrupted (all criteria met)
3	Greyish-Green	At least one criteria but not all criteria met
4	Yellow	Elimination policy being implemented
5	Blue	Priority for epidemiological surveys
6	Red	Low priority area for epidemiological surveys

2.4 Rationale for adoption of onchocerciasis elimination policy.

The annual treatment with ivermectin has been the main strategy used for onchocerciasis control/elimination in Uganda. Although more than 70% of endemic communities have received an annual dose of ivermectin for at least 10 years, with a significant reduction in the prevalence of onchodermatitis, nodules and microfilaria (Ndyomugyenyi, *et.al.* 2007; Katabarwa, *et.al,* 2008) but the same communities still have *O. volvulus* parasites in their skin ranging from 1.5-27.8% (MOH, 2007). Studies conducted in Itwara focus in western Uganda demonstrated that annual treatment alone was not able to interrupt transmission (Garms, *et. al,* 2009). Winnen *et al.,* 2002 also observed that annual ivermectin treatment required to eliminate *O. volvulus* in areas with medium to high levels of endemicity with treatment coverage of 65% or 80% is estimated at 40 years and 25 years, respectively.

In Uganda, the combination of annual treatment and vector elimination has been shown to be quite effective in drastically reducing the burden of the disease (Ndyomugyenyi, *et al.*, 2004). Vector elimination is backed by the fact that most of the foci are not only isolated but the vector species (especially *S.neavei*) have a short flight range and are very vulnerable and easily disappears due to insecticidal pressure or environmental degradation (Mpagi *et al* 2000; Raybould & White 1979; Walsh, *et al.*, 1996).

In view of the above and aware of its commitment to onchocerciasis elimination in Uganda, the MOH adopted the onchocerciasis elimination policy whereby semi-annual CDTI combined with vector elimination strategy would be used instead of annual CDTI in order to achieve interruption of transmission and eventually certifying the country free of the onchocerciasis.

3.0 CERTIFICATION CRITERIA FOR ONCHOCERCIASIS ELIMINATION.

The certification of onchocerciasis elimination in any focus in Uganda will be based on i) the elimination of morbidity and; ii) interruption of transmission. These parameters will be assessed and measured using standard procedures and techniques (WHO, 2001). The verification of claims of elimination of onchocerciasis in a defined focus will be done meticulously. It is therefore important that all information pertaining to the elimination campaigns be documented, archived and made available for verification by both national and international certification teams. The quality of the data will also be examined and where the teams will have not been satisfied with quality and validity of data, an expert opinion may be sought or re-evaluation be instituted by an independent team or consultant.

In particular, data on ivemectin treatment, health education campaigns, and vector control/elimination should be available at least at higher levels of surveillance systems. This information will constitute an important part of the exercise. Assessment of community awareness about the disease and interventions implemented will also be evaluated.

Verifiable bench marks or indicators have also been developed for active assessment of proof of interruption of transmission in a defined focus. The interruption of disease transmission will be assessed and determined based on epidemiological and entomological criteria. These indicators comprise of the elimination of morbidity, absence of detectable *O. volvulus* antibodies, absence of crabs and / or infestation with *S.neavei* larvae/pupae, and infection rates in *S. damnosum sl.*

3.1 Elimination of morbidity

The assessment of elimination of morbidity will be based on microfilaria prevalence in skin snips (see Appendix 3), considered to be a proxy indicator for skin and eye lesions due to onchocerciasis. The threshold of morbidity elimination as defined by Diawara, *et.al* (2009) criteria is considered achieved when the microfilaria prevalence in skin snips in less than 5% in all sampled communities and less than 1% in 90% of sampled communities. In foci where ocular disease may occur, presence of microfilaria in anterior chamber or in cornea will be conducted using slit lamp procedure. The goal is to achieve a prevalence of less than 1% before halting interventions. (WHO, 2001; Lindblade *et al.*, 2007)

3.2 Epidemiological criterion.

This criterion is defined by the absence of detectable infection (as evidenced by microfilaria, immunological tests) in young children (aged less than 10 years). The *O. volvulus* antibody test developed by Lobos *et al* (1991) using the OV-16 antigen using finger prick blood samples in ELISA assays (see Appendix 6) is recommended as a primary screening tool. A 5-year cumulative incidence of less than 1 new case per 1000 (0.1%) is acceptable (WHO, 2001). A sample size of 3000 is needed to statistically exclude 0.1%. Sampling will be representative of the entire focus and at least 3000 children under 10 years will be examined unless that number does not reside in the entirety of the focus. Putative positives can be confirmed using O-150 PCR with skin snips to demonstrate actual infection (Meredith, *et. al.*, 1991).

3.3 Entomological criterion.

Entomologic assessment of interruption of transmission will depend of the disease vector species as follows:-

3.3.1. *S. neavei* infested foci.

In the *S. neavei* foci crabs and / or fly collections will be used. The lack of positive crabs for larvae/pupae of *S. neavei* species in a series of surveys (see Appendices,5a,b) and the absence of flies collected in a defined focus over a period of 3 years is indicative of interruption of transmission (Garms *et al.*, 2009).

3.3.2 *S. damnosum s.l.* infested foci

In the *S. damnosum s.l.* infested foci, the infection rates of the vectors as determined by dissections and PCR will be used. The *Simulium* flies will be collected during the hours of the day when parous flies are most abundant during the peak transmission season (WHO, 2001; see Appendix 4). ATP of zero if interruption and elimination achieved, infection in flies <1/1000 in parous flies, L3 in flies <0.05% assumes 50% nulliparous rate and ATP or STP lower than 5-20 L3/person. Interruption of transmission will have been achieved when infection rates in flies as determined by O-150 Poolscreen PCR is less than 0.05% in a sample of 10,000 flies per focus (see Appendix 7).

4.0 PHASES OF ONCHOCERCIASIS ELIMINATION PROCESS.

Before any onchocerciasis endemic focus can be certified free of transmission, four phases of the process has to be achieved as follows:-

a) Pre-intervention phase

- Identify and stratify all endemic communities and establish sentinel village(s) in each focus
- In sentinel communities carry out in depth epidemiological surveys for base line.
- Carry out baseline entomological studies

b) Intervention phase

- Initiate semi-annual treatment of all endemic communities targeting 90% coverage of the eligible population.
- Establish and maintain semi-annual treatment in all foci defined for elimination
- Initiate and maintain larviciding activities in the focus, if appropriate.
- Conduct monitoring and evaluation activities as appropriate

c) Interruption of transmission / halting of ivermectin treatment

- UOEEAC reviews all available data pertaining to the focus to ascertain if transmission is interrupted, referring to the criteria established in this document.
- If the criteria are met, UOEEAC recommends to government in writing that ivermectin treatment cease.
- NCC reviews recommendation and makes decision. Consultation with MOH, Districts, Partners, and Chair UOEEAC.
- If the decision is to stop ivermectin, health education (information, education, communication) and consultation will take place in communities to advise prior to halting treatment
- The programme will maintain ivermectin tablets for clinical treatment of onchocerciasis cases as needed, based at frontline health units.
- A three year post-treat surveillance (PTS) period will begin to determine if recrudescence occurs. Heightened surveillance activities will be maintained during this period.
- Onchocerciasis elimination verification surveys may be carried out at any time during this process, under the auspices of the NCC and preferably by an independent team.

d) Post-Treatment surveillance

- Ongoing post treatment surveillance (PTS) will be implemented for a period of three years by MOH staff and its partners.
- After, an evaluation will be conducted by an independent team under the auspices of the NCC to avoid conflict of interest.
- Data from surveillance and independent evaluations will be submitted to the NCC. NCC will review the recommendation and make a decision. If no recrudescence is noted then the focus will be declared by the NCC to have been eliminated.

4.1 Post Treatment Surveillance (PTS)

The concept of post treatment surveillance adapted from the WHO document on "Certification of elimination of human onchocerciasis: Criteria and Procedures" in which a three year period was described as "Pre-certification Period" (WHO, 2001). This was a national period "(i.e., related to the entire country rather than individual onchocerciasis foci) during which surveillance was instituted after intervention were halted to detect recrudescence of transmission of *Onchocerca volvulus*.

"With the ceasing of interventions, a 3 year pre-certification period would start. At the end of this pre-certification period, it must be shown that, although intervention has ceased, no new incidences of onchocerciasis cases have been registered and no infected vectors identified (WHO, 2001). Based on this original WHO statement, the UOEEAC should address both the ***"foci issue"*** and the ***"country issue"***. It is imperative to note that a meeting held by WHO Certification committee in 2007 recognized the importance of data from Uganda as far as improving the current WHO Certification guidelines based on the Guinea worm eradication and onchocerciasis elimination in the Americas. Therefore, UOEEAC should appropriately modify the 'pre-certification period' definition so that it could be applied to foci as well, rather than entire country. PTS should put into account halting of ivermectin treatment and vector elimination or targeted vector control where applicable. PTS could be defined as follows:

- A 3-year period that begin with cessation of larviciding. At end of this period, no vector *Simulium* flies or positive crabs should be caught.
- A 3-year period that begins with termination of mass treatment, no infected vector flies with larval stages of L1s, L2s and L3s in *S.neavei or S. damnosum* should be detected.
- A 3-year period that begins with the termination of mass treatment with ivermectin for onchocerciasis. At the end of this period, it must be shown that, although intervention has ceased, no evidence of recurrent transmission has been documented based on vectors in some foci and assessed children.
- If the data indicated no recrudescence of *Onchocerca volvulus* transmission it should be declared eliminated.
- Post elimination (also termed 'post endemic) surveillance (PES) should also continue in formerly endemic foci beyond the initial 3 years PTS.

4.1.1 PTS Procedures

The procedures for PTS recommended by WHO (2001) should be based on the consideration that PTS should use the same sampling and diagnostic techniques for foci with similar interventions in order to decide that transmission of *Onchocerca volvulus* had been interrupted. However, PCR-skin snips and OV16 will follow the standard protocol for all the foci (see Appendices, 7 & 8). This is required to enable UOEEAC provide appropriate recommendation to government on the stopping of intervention in a given endemic focus. These procedures are provided with certain fundamental consideration in mind:

a) PTS should not be completely new to the programs but continuation of previous field activities.
b) Indices would therefore be comparable and initial survey would serve as baseline data.
c) PTS should take advantage of the longitudinally followed sentinel villages where the baseline data exist.

In terms of timing of these evaluations, it is recommended that;

- Entomological surveillance is conducted similar to that done in pre-PTS surveys.
- The OV16 antibody survey of children under ten years of age may be conducted in year 3 of PTS with confirmation of positives using PCR-skin assays in year 3 of PTS.

4.1.2 District and Community sensitization

The district authorities and the communities should be sensitized prior halting intervention in any focus, so that they are prepared to take required roles in the Post-Treatment surveillance. The district authorities need to be alerted on strengthening surveillance at all levels. The fears for any recrudescence or resurgence will be counteracted through intensified community sensitization.

Post-Treatment surveillance is one of the critical interventions to be implemented during the three years preceding the certification process. Therefore, it is important that the national surveillance system must be strengthened at all levels to ensure onchocerciasis detection and appropriate actions. In foci under vector elimination, the associated vectors should be monitored both in the catching and breeding sites. Therefore the surveillance system should:-

- Enhance the sensitivity about onchocerciasis by the national health care system including the communities by maintaining a high degree of public awareness of onchocerciasis and need for immediate treatment with ivermectin
- Maintain a village based surveillance in all formerly onchocerciasis endemic foci supported by VHTs, parish/community supervisors and CDDs.
- Incorporate surveillance of other diseases into the surveillance system for onchocerciasis or vice-versa.
- Ensure periodic surveys to monitor recrudescence or vector re-invasion of previously free foci.
- Ensure monitoring and evaluation of community awareness about the disease and involvement in the implementation of intervention in the defined foci.
- Maintain proper documentation of all activities and reports which must be archived and stored properly at all levels (National, district, Sub-county, and community levels for ease of verification by NCC and ICT)

5.0 UGANDA ONCHOCERCIASIS CERTIFICATION COMMITTEES

5.1 Uganda Onchocerciasis Elimination Expert Advisory Committee (UOEEAC)

The Uganda Onchocerciasis Elimination Committee (UOEC) now renamed Uganda Onchocerciasis Elimination Expert Advisory Committee (UOEEAC) was formed in 2008 with the objective of providing technical advice to the Ministry of Health on onchocerciasis elimination in the country. The members of this committee are composed of nationals and international experts who are involved in reviewing progress annually in both the control and elimination activities.

All foci attaining the above mentioned indicators will be presented by the elimination programme to the Uganda Onchocerciasis Elimination Expert Advisory Committee (UOEEAC) meeting which will sit once a year in Uganda, and determine with available data whether interruption and elimination of transmission has been attained. UOEEAC will then recommend to move the concerned foci from the yellow band of the "oncho flag" to the light green where post-treatment surveillance (PTS) will commence. The UOEEAC meeting may also recommend to the government

through Ministry of Health to halt all interventions. It is the responsibility of MOH through the NCC (see below) to approve halting of interventions and ensure that concerned district authorities are informed about the decision and the programme educates affected communities about the decisions made.

5.2 National Certification Committee (NCC)

The National Certification Committee (NCC) focuses on programmatic issues within the Ministry of Health that are outside the mandate of the UOEEAC. The NCC will also be the locus of a technical review of the recommendations provided by the advisory committee (the UOEEAC) in order to enable the Ministry of Health to take prompt and appropriate decisions on those recommendations. The NCC will review such recommendations in light of the Health Sector Strategic Investment Plan (HSSIP). The NCC will also prepare the country for certification of onchocerciasis by the World Health Organisation.

6.0 National preparation for certification

National preparation activities leading to certification should be carefully planned and implemented to ensure that items needed are in place by the time the international certification team is invited in the country. Annual work plans should be set priority activities to be implemented during the pre-certification period as follows:-

6.1 Preparation of reports

Since the attainment of certification level will be achieved at different times in the various onchocerciasis endemic foci, there will be two kinds of reporting. Firstly detailed report will be compiled on every focus that has attained certification status as verified by the National certification committee. Secondly, when all the different foci have attained certification status, a final report will be compiled for the whole country by the NCC. These reports may vary but must be verifiable, factual and evidence based and the contents of which should include the following:-

- Historical overview of the onchocerciasis in the foci / country and detailed implementation of the elimination campaigns including challenges and lessons learnt during the campaigns
- Overview of the administrative system and health care infrastructure and delivery system as well as the capacity to detect and manage cases should such occur in future.
- A critical review of the threat of recrudescence or re-invasion by vectors in previously freed foci from the neighbouring foci or countries.
- An evaluation of the effectiveness of the routine disease reporting system, and how the system could capture information on onchocerciasis should such cases occur in the future and how feed back information could be passed.
- The report should use maps and graphs to illustrate changes in the disease trends and distributions as well as for health / administrative infrastructures in the country.
- The report should present coherent recommendations on post certification interventions to check recrudescence or re-introduction of the disease in the freed foci or country as a whole.

When the country report has been compiled and validated by NCC, the members of the NCC including the Secretariat must sign it before submitting to the Permanent Secretary / Ministry of Health for adoption and subsequent submission to the WHO country office. Based on the country report and the recommendations of the NCC, the Minister of Health will sign a declaration to the effect that indeed onchocerciasis has been eliminated in Uganda and will invite the ICT to come to Uganda to verify the report and have Uganda declared free of onchocerciasis.

7.0 International Certification Team

The International Certification Team verifies submitted reports through field visits and reviews documents at all levels and makes a report to WHO recommending certification or not. It is therefore important that the country report must be factual, verifiable and evidence based. Preparations at all levels must be thorough and complete and relevant documents must be made available before their arrival since they will have limited time in the country.

8.0 CONCLUSION

The preparation of the country for certification of onchocerciasis elimination is a multi-faceted process involving many stakeholders. Hence the development of a common tool to guide the process to the realization of the goal within a period of time is critical. It is envisaged that the development partners especially the World Health Organization will continue to provide technical support in form of consultants from time to time not only to strengthen the national capacity but steer the process of preparedness for certification. The Ministry of Health remains committed to ensuring that once eliminated, onchocerciasis does not re-establish itself again in Uganda.

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APPENDIX 1: ONCHOCERCIASIS ELIMINATION FLAG FOR UGANDA

Note

Colour	Meaning
Dark Green =	Eliminated
Light Green =	Transmission Interrupted
Greyish Green =	Interruption suspected
Yellow =	Implement elimination policy
Blue =	Priority for epi studies for delineation of each focus before semi-annual treatment decision
Red =	Not much is known (Need for Epi stidies)

Uganda's plan for onchocerciasis elimination (August 11, 2010)

ID No.	Focus	PTS Ye/No	Vector	District	# MDA annual rounds	# of MDA semi annual rounds	Total Pop	2010 UTG1	UTG2	Status of Transmission	Yr of elimination	Plan for MDA treatment	Plan for Larviciding	Larviciding Start/End
1	Victoria		S. damnosum	Jinja	N/A	N/A	198,160			Eliminated	1973	No need	No need	
				Mukono	N/A	N/A	387,707			Eliminated	1973	No need	No need	
				Kamuli	N/A	N/A	268,046			Eliminated	1973	No need	No need	
				Mayuge	N/A	N/A	156,714			Eliminated	1973	No need	No need	
				Kayunga	N/A	N/A	142,565			Eliminated	1973	No need	No need	
2	Wadelai		S.neavei	Nebbi	15	9	17,739	14,382	28,764	Interupted		Semi-Annual	not done	No need
4	Itwara		S. neavei	Kabarole	20	N/A	30,689	25,655		Interupted		Annual	Status post	2003
				Kyenjojo	20	N/A	63,850	50,823		Interupted		Annual	Status post	2003
5	Mt. Elgon		S.neavei	Manafwa	15	7	39,231	32,558	65,116	Interupted		Semi-Annual	Vector Elimination	2008
				Mbale	15	7	49,264	40,173	80,346	Interupted		Semi-Annual	Vector Elimination	2008
				Sironko	15	7	75,016	63,247	126,494	Interupted		Semi-Annual	Vector Elimination	2008
				Bududa	15	7	156,164	134,933	269,866	Interupted		Semi-Annual	Vector Elimination	2008
3	Mpamba-Nkusi		S. neavei	Kibale	17	N/A	180,287	149,356	298712	Interruption Suspected		Annual	Status post	2006
6	Imaramagambo		S.neavei	Bushenyi	18	N/A	102,180	85,748		Interruption Suspected		Annual	not done	No need
15	Nyamugasani		S.damnosum	Kasese	17	N/A	11,270	10,167		Interruption Suspected		Annual		
7	Kashoya-Kitomi		S.neavei	Buhweju	16	7	130,855	107,830	215,660	uncertain		Semi-Annual	Vector Elimination	2007
				Rubirizi						uncertain		Semi-Annual		
				Ibanda	16	7	24,778	20541	41,082	uncertain		Semi-Annual	Vector Elimination	2007
				Kamwenge	18	7	41,021	33,831	67,662	uncertain		Semi-Annual	Vector Elimination	2007
8	Wambabya-Rwamarongo		S. neavei	Hoima	16	7	72,077	60,918	121,836	ongoing		Semi-Annual	Vector Elimination	2009
9	Budongo		S.neavei	Masindi	16	7	44,763	37,227	74,454	ongoing		Semi-Annual	Vector Elimination	pending
				Bulisa	16	7	25,139	21,595	43,190	ongoing		Semi-Annual	Vector Elimination	pending
				Hoima	16	7	73,069	61,326	122,652	ongoing		Semi-Annual	Vector Elimination	pending
10	Bwindi		S.neavei/ S. damnosum	Kabale	15	7	27,604	22,499	44,998	ongoing		Semi-Annual	Vector Control	pending
				Kanungu	15	7	54,416	44,242	88,484	ongoing		Semi-Annual	Vector Control	pending
				Kisoro	15	7	35,141	28,894	57,788	ongoing		Semi-Annual	Vector Control .	pending
	Mid North I			Pader	N/A	N/A	450100	373583		ongoing		Annual		
				Kitgum	N/A	N/A	133888	111128		ongoing		Annual		
				Lamwo						ongoing				
				Amuru						ongoing				
				Gulu						ongoing				
				Lira						ongoing				
12	Nyagak Bondo		S.neavei	Nebbi	18	N/A	653,645	581,197		ongoing		Annual		
11	Maracha Terego		S.neavei/S.damnosum	Maracha Terego	18	N/A	170,377	136,302		ongoing		Annual		
13	Obongi / Moyo		S.neavei	Moyo	17	N/A	41,182	33,368		ongoing		Annual		
14	Lubilila		S.damnosum	Kasese	17	N/A	119,315	102,718		ongoing		Annual		
16	Madi		S.damnosum	Moyo	17	N/A	113,106	91,290		ongoing		Annual		
				Adjumani	17	N/A	164,780	140,208		ongoing		Annual		
17	West Nile		S.neavei/ S. damnosum	Yumbe	18	N/A	286,615	229,292		ongoing		Annual		
				Koboko	18	N/A	167,076	133,661		ongoing		Annual		
				Arua	18	N/A	138,063	134,696		ongoing		Annual		
18	Mid-North		S.damnosum	Oyam	17	N/A	20,345	17,011		ongoing		Annual		
				Gulu	17	N/A	117,510	94,872		ongoing		Annual		
				Amuru	17	N/A	151,098	120,145		ongoing		Annual		
	Total				535	107	5,134,845	3,345,416	1,747,104					

ɂdjumani districts reduced due to repatriation of Refugees back to Sudan

APPENDIX 2: DEFINITIONS RELEVANT TO ONCHOCERCIASIS ELIMINATION

An **onchocerciasis** case is defined as an individual with evidence of current infection with *Onchocerca volvulus*.

Case definition: In an endemic area a person presenting with fibrous nodules in subcutaneous tissue. Laboratory confirmation are the presence of microfilaria in skin snips taken from iliac crest, presence of adult worms in excised nodules and presence of ocular manifestations, such as slit lamp observations of microfilaria in the cornea, the anterior chamber or vitreous body.

Incidence is the rate at which new cases arise in a population within a defined interval.

Prevalence is the proportion of the host population infected at a particular point in time.

Morbidity is defined as the presence of a disease manifestations caused by onchocerciasis.

Interruption of transmission means the permanent interruption of transmission in a clearly defined area (focus) after all the adult worms in the human population in that area have either died out from old age or been exterminated by some other intervention. This should occur within 15 years of the establishment of sustained interruption of infectivity.

Sentinel communities are preselected hyperendemic communities where in-depth epidemiological evaluations take place at regular intervals; first before treatment starts, then again after two years and finally at 4-year intervals thereafter. The evaluations include parasitological (mf and nodules), ophthalmological, and entomological indicators. It should be noted that the use of sentinel communities in this way has two disadvantages. First, the community populations may become tired of these repeated examinations and refuse to cooperate. Second, it will soon become known by those working in the programmes which are the designated sentinel communities and they may reserve their best efforts for these communities at the expense of others. A possible way round this difficulty is to have a larger number of potential sentinel communities and just before each round of examinations to pick at random a smaller number of them that will be examined. The International Certification Team is encouraged to use other villages for monitoring, pre-certification or certification activities.

Elimination (literally "casting out over threshold) of the parasite population from a defined geographical area means the sustained absence of transmission until the adult parasite population within that area has died out naturally or has been exterminated by some other intervention. This should occur within 15 years after interruption of transmission. When elimination of the parasite is certified, the endemic area moves into the "post endemic" phase.

Pre-certification period is the period following interruption of transmission, during which surveillance is carried out to verify that interruption of transmission has been sustained after ceasing control interventions. This period last for 3 years and no intervention is carried out.

Certification: a country will be eligible for certification as being in post endemic phase after successfully completing a 3-year pre-certification period in all its foci.

Endemic: when onchocerciasis morbidity, transmission and infection are present.

Post-endemic: When a country with a past history of endemic onchocerciasis is officially certified as having successfully completed a 3-year pre-certification period of interrupted transmission in all its previously endemic onchocerciasis foci.

Endemicity is the permanent presence of the disease or pathogenic agent in a given region. Its level is determined according to the presence of the disease or pathogenic, i.e. the percentage of diseased persons or carriers in a given population.

An endemic onchocerciasis focus: is an area within a country where a local cycle of Onchocerca volvulus transmission is maintained and is giving rise to autochthonous infections. In terms of population biology of the parasite, this is an area where the basic reproductive ratio (Ro) is 1.0 or

greater. Endemicity is stable where the incidence of the infection shows little or no trend to increase or decrease over time.

Geographical coverage: meaning percentage of endemic communities receiving ivermectin. The requirement being that all endemic (100%) communities be identified and receive regular mass distribution of ivermectin.

Therapeutic coverage: meaning percentage of total population of endemic community treated. The requirement being at least 80% of the total population of an endemic area.

Programme coverage: meaning percentage of eligible population treated. The requirement being at least 95% of the eligible population in each community to be treated at each round.

APPENDIX 3: STANDARD OPERATING PROCEDURES FOR SKIN SNIP FOR *ONCHOCERCA VOLVULUS* PARASITE

Preparation for Skin snip exercise:

Community assessment for onchocerciasis infection by skin snip should be well planned and communities mobilized in advanced of planned survey in order to enlist their cooperation and participation. Rainy season should be avoided since it may not only make it difficult to access some villages but people may be busy in their fields contributing to poor turn up.

Procedure of conducting skin snip

Before taking a skin snip, ensure that there is privacy for participants. The selected site for skin snip is usually the iliac crest; ensure that this is swabbed with alcohol pad. Remove a small bloodless fragment of skin from the iliac crest area, using a scapel or razor blade for each participant. The skin biopsy is placed in 0.1 normal saline in a microtitre plate. It is allowed to incubate for 24 hours (WHO, 1995), and examined under a compound microscope for microfilariae. In the event of a positive test, one observes the free microfilaria under the binocular microscope. The microfilaria is enumerated in each skin snip and from there prevalence, density, and Community microfilariae loads are calculated (WHO/APOC, 2002).

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APPENDIX 4: STANDARD OPERATING PROCEDURES FOR HUMAN LANDING CATCHES IN SIMULIUM ADULT FLIES

Choice of catching point

Site selection as catching points is critical and should be based on the following criteria and should preferably be situated close to sentinel communities identified for periodic epidemiological studies. The criteria for selection include:

- The catching point should be located in a zone where the conditions of transmission of infection are met: presence of man, parasite and vector.
- It should be near a significant and productive breeding site.
- It should be in a shade and sheltered or protected.
- It must be accessible all the year round.
- It should not be a place of human gathering, in order to avoid at the same time dilution and a drop in the vigilance of the catchers.

Catches on humans (human landing catches)

The catches made on humans make it possible to know the density of the population of biting females of *S. neavei or S. damnosum s.l.* , in a given place and period, and to estimate it in number of bites/man/day. The use of man as bait is common for catching many hematophagous insects.

Collector teams

Each catching team is made up of two people who carry out catches of *simulium* for one hour in turns. It is known that the *S. neavei/ S. damnosum s.l.* bite, preferably the lower limbs. Taking into account this low location of the bites of females, only the legs of the catcher are stripped and exposed. The black flies that alight on him are immediately covered, then trapped in catching tubes, on a rate of one fly per tube. The black flies thus caught are, in theory, meant for a study of their infection by *Onchocerca volvulus*. Thus it is absolutely necessary that the females, which come to bite, be caught before they start their process of biting, in order to avoid loss of parasites, and the infection of the catcher.

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APPENDIX 5a,b: STANDARD OPERATING PROCEDURES FOR CRAB CATCHING AND EXAMINATION IN *S. NEAVEI* GROUP

Section 5a. Crab catching and examinations are always done to determine the infestation of fresh water crabs. The immature stages (larvae and pupa) of all vector species live attached to fresh water crabs (*P. aloysiisabaudiae, P. niloticus, P. loveni)* in phoretic association. Phoretic rates of more than 50% have been reported in a number of foci in Uganda (Barnley, 1975). Locally made basket traps are used in crab catches using following procedures:

Camouflaged basket traps baited with fresh red meat are placed in appropriate sites along a river course; most preferable stony with not very fast flowing water should be selected for placing the trap. Traps are always placed with the side opening facing towards the current, to allow water to carry the scent of the bait some distance away from the trap. The duration of trapping is one hour (Garms, *et.al*, 2009), but in places where crabs are few it can be left over night. Later, the traps are removed and crabs are emptied in a container with clean water. The crabs are then identified, carapace size measured with vernier calipers and carefully examined for larvae or pupae of *S. neavei.* Number of immature stages are counted and then recorded in crab form. The GPS locations of sites where catches are conducted are always recorded.

Section 5b. Where there are no pre-control data confirming the species of vector it has to be assumed that either vector may be involved. Therefore, surveys must be directed at both *S. damnosum s.l* and *S. neavei*, until evidence confirms which vector is involved. Where the vector is a member of the *S. damnosum* complex surveys should be followed as outlined in section 3.3.2 and appendix 7 of this document (WHO 2001).

Where the vector is *S. neavei* entomological surveys should primarily employ trapping and examination of crabs for infestation with immature stages of *S. neavei.* This is more sensitive and less costly than human landing fly catches. It has no adverse, ecological consequences, as the crabs are returned unharmed to their breeding rivers. Crab trapping must be carried out at least twice annually, at each site, for three years, a minimum of two traps to be used on each occasion. This is sufficient to establish the presence or absence of crabs and infestation.

It is not possible to specify the number of necessary trapping sites, as this will depend on the size of the focus and the complexity of the hydrological network. Coverage must be comprehensive, which means at least 3 sites / medium sized river (one each in upper, middle and lower reaches). Such sites should not be more than 10 km apart. Small streams may be monitored by single trapping sites. Where a survey shows the presence of infested crabs, detailed investigations (including human landing captures) may be carried out.

There is clear evidence that *S. neavei* takes 18-36 months to recover from local elimination. Therefore, it is recommended that the 3 year post larviciding surveys should only begin 1 year after the cessation of treatments.

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APPENDIX 6: STANDARD OPERATING PROCEDURES FOR OV-16 ELISA ASSAY

Introduction

Inoculation of third larval stages of *Onchocerca volvulus* into the humans elicits body immune response. The specific anti-body iso-type is IgG4 which is detected in blood by a specific antigen, OV-16 using the OV16 ELISA assay. The technique is a very sensitive tool which detects the lowest levels of Onchocerciasis transmission

1.1 Purpose

The dry blood spots are eluted and examined to determine the exposure of children to infection in onchocerciasis endemic areas where the disease elimination has been launched. The technique is a sensitive epidemiological tool for monitoring and evaluation of *O. volvulus* elimination programme. The presence of antibodies specific for the antigen is an indicator of exposure.

1.2 Specimen required

Finger prick blood collected on Whatman filter paper #2. The dry filter paper sample must be stored at -20 degrees Celsius.

1.3 Materials and reagents required

1.3.1 Sample Collection

Cotton wool
Disinfectant solution
Sterile single use lancets
5 x 5cm whatman filter paper#2
Plastic ziplock envelopes
Cool box and ice packs
Pencils
Drying rack
Sharps disposal box

1.3.2 Sample Elution

Punching machine
6mm punch of the sample spot
96 well plastic ELISA plates
5% Bovine serum albumin
Multi-channel pipette (P -200)
P-200 tips
Elution is done at 8 degrees Celcius overnight

1.3.3 Materials for the ELISA procedure

96 well 2HB ELISA plates
OV-16 antigen in coating buffer)
Vortexer
Monoclonal anti human IgG4 biotin conjugates
Streptavidin- AP (Invitrogen 19542018)
P-Nitro phenyl phosphate (Pnpp) solution (sigma N-9389)
Standard serum
Positive and negative controls
3M sodium hydroxide solution
gloves

P1000, P 200, P20, P10 automatic pipette with respective tips
Sample maps
Plate cover tapes
A-4 zip lock plastic bags
Washing buffer with tween- 20
Absorbent tissue paper
ELISA plate reader

1.4 ELISA procedures

-Punch out two 6mm spots from the main dry blood sample and place into the 96 well elution plates. Label the position of each sample in the plate using a sample map.
-Add 200ul of 5% BSA to all sample wells and keep at 2-8 degrees Celsius overnight.
-Coat 2HB 96 well assay plates with 1Um OV 16 antigen diluted in carbonate buffer and keep at 2-8 degrees Celsius overnight. Read 405nm every minute until standard 1/40 is OD 1.15. stop reaction with 25ul 3M NaOH
BLOCKING: Add 100μl PBST-BSA to each well.
Incubate for 1 hr at 4°C. After 1hr dry the plate, do not wash
ADDING STANDARD AND CONTROLS: Control: 50 μl, 1/50 dilution with PBST-BSA 5% Standard: 5
Incubate for 2 hours and wash each plate 4 times with 10XPBST washing buffer.
CONJUGATE: Monoclonal anti-human IgG4 clone HP-6025, conjugated to biotin. Dilution: 1:15000 in PBST. Add 50 μl to all wells and incubate for 1 hour at room temperature. Wash each plate 4 times with 10x PBST washing buffer.
STREPTAVIDIN-AP: dilutions, 1:2000 in PBST. Add 50μl to all wells. Incubate for 1 hr at room temp in a zip lock .Wash each plate 4 times with PBST washing buffer.
SUBSTRATE: 1mg/ml of pNPP in substrate buffer. Add 50ul all wells. Leave plates to develop at room temperature.
When the Optic density (OD) of the first standard is 1.15, stop the reaction by adding 25μl of 3M NaOH to each well.
Read the plate using an ELISA reader at wavelength of 405nm.

1.5 Reporting

The results are given as positive or negative. Samples which give OD equal or greater than 40 are positive while those with less than 40 are negative.

1.6 Quality control

All putative positive samples must be repeated to confirm the positivity.
The standard curve of concentrations of standard sera and control sera must be within the acceptable ranges given in the quality analysis calculations.
Repeat all positive samples with GST and OV16 to rule out false positivity due to the presence of GST in blood of children.

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APPENDIX 7: STANDARD OPERATING PROCEDURES FOR O-150 POOL SCREEN PCR FOR *SIMULIUM* FLIES

1.1 Introduction

In Uganda Onchocerciasis is transmitted by *S. neavei* and *S. damnosum*. The third stage infective larvae are found in the head of the vector. The transmission rate of the parasites by the vector can be determined by molecular methods. O150 PCR is a molecular epidemiology technique which detects a tandemly repeated 150 base pair DNA segment of the parasite in the vector.

1.2 Purpose

For monitoring *O. volvulus* transmission by *Simulium* vector flies.

1.3 Specimens Required

Adult *Simulium* vector flies caught using the human bait method.

1.4 Materials and reagents required

1.4.1 Materials for DNA extraction and purification

1.5 ml Snap cups, 95% Ethanol, pipette tips, pasture pipettes, NaCl, Tris HCl, EDTA, SDS, Carrier DNA solution, plastic homogenizers, protenasek solution, heating blocks, thermometer, DTT, freezer, phenol, chloroform, NaI, 96 well filter plates, vacuum pump, Ethanol wash, Elisa plates, multi- adapter centrifuge, distilled water

1.4.2 Materials for DNA amplification

PCR water, PCR tubes, DNTPs, taq polymerase, primers, buffer, sample DNA, pipettes and respective tips, positive controls, PCR thermocycler

1.4.3 Materials for DNA Detection

Elisa plates, coating buffer, streptavadine [Jackson Immuno Research], incubators, hybridization buffer, antifluoresceine, fab fragment, antibody dilution buffer, OVS-2 FL probe, SSPE / SDS buffer, BRL substrate, BRL amplifier, sulfuric acid.

1.5 Methods

1.5.1 Preparation of DNA from Pools of Heads or Bodies

1. Make pools of 50 flies in tubes.
2. Place the tubes containing the 50 flies in liquid Nitrogen to make the flies brittle. Remove the tubes; shake them vigorously to break the heads from the bodies.
3. Place the heads or bodies in a 1.5ml snap cap microcentrifuge tube. Rinse the flies three times in 95% ethanol. Remove as much of the ethanol as possible using a narrow tip pipet, or pipetman.
4. Allow the ethanol to evaporate for about 10 minutes at room temperature.
5. Add 300ul of homogenization buffer to the tube containing the flies. To the mixture, add 2µl of carrier DNA solution (250ng/µl salmon sperm DNA). Homogenize with a plastic homogenizer. Homogenize until the flies are completely broken up.
6. Add 100ul of proteinase K solution (400ug/ml prepared in homogenization buffer; make fresh each day).
7. Incubate the extract at 55°C for one hour.
8. Add 4µl of 1M DTT. Transfer the extract to a screw cap tube, and boil for 30 minutes.
9. Freeze-thaw the extract two times.
10. Extract the homogenate with 400µl of a 1/1 mix of phenol and chloroform. Transfer the top layer to a second tube, and perform a second extraction with phenol/chloroform.
11. Extract the aqueous layer from the second phenol extraction one time with 400µl of chloroform.

12. Measure the volume of the aqueous layer. Add 3 volumes of NaI solution. Incubate in the refrigerator for 15 minutes.
13. Place the 96 well unifilter (Whatman GF/B or GF/C) over the pump unit and carefully apply the solution from step 11 to each well. Switch on the vacuum pump. After all the solution has been drawn through the filter, switch off the vacuum pump.
14. Add 500µl of ethanol wash to each well and switch on the vacuum pump. After all the ethanol wash has been drawn through the wells, switch off the vacuum pump.
15. Repeat step 13 twice.
16. Place the filter over the 96 well collection plate; add 50µl of sterile distilled water at 55°C to each sample. Centrifuge the unit (filter+ plate) at 4000 RPM for 1 min.
17. Add 150µl of NaI solution to each well, and incubate in the refrigerator for 15 minutes.
18. Repeat steps 12-14. Elute the DNA from the plate into 50µl of sterile distilled water, as described in Step 15.
19. Store the DNA at -20°C.

1.5.2 Amplification Procedure

1. Prepare a master mix in a clean room.
2. Add 5 µl of the DNA to each well of the master mix plate in the DNA preparation room.
3. Place the plate in a PCR machine and amplify using the following **PCR program**:

5 cycles:
1 minute @ 94°C
2 minutes @ 37°C
30 seconds @ 72°C
Then go to 35 cycles:
30 seconds @ 94°C
30 seconds @ 37°C
30 seconds @ 72°C
Finish with 6 minutes @ 72□C

1.5.2 Detection of PCR products

This is done using a PCR ELISA:
1. Coat plates with 100□l of 1□g/ml Strepavidin for a minimum of 2 hours @ 37°C. or 4°C overnight.
3. Wash plates 6 times with TBS/Tween, emptying the plate on to a paper towel between washes.
4. Add 20□l hybridization buffer (HB) to all wells.
5. Add 5□l of undiluted PCR product (10% of the PCR reaction) to the appropriate wells.
6. Incubate 30 minutes at room temperature.
7. Wash the plates 6 times with TBS/Tween.
8. Add 100□l 1N NaOH to all wells.
9. Incubate for 1 minute at room temperature.
10. Wash plate 6 times with TBS/Tween.
11. Add 50 □l OVS2-FL-probe diluted to 50ng/ml in HB to all wells.
12. Incubate for 15 minutes at 42°C.
13. Wash the plate 6 times with TBS/Tween.
14. Add 100□l pre-warmed SSPE/SDS buffer to all wells.
15. Incubate 10 minutes at @ 42°C. Remove substrate and amplifier reagents from freezer if already made up or fridge if they need diluting and let come to room temperature. Prepare the conjugate (anti flouresecin Fab fragment) by diluting it 1/10,000 in antibody dilution buffer (i.e. 1□l Fab fragment plus 10ml dilution buffer).
16. Wash the plate 6 times with TBS/Tween.
17. Add 50 □l of the diluted anti-florescin Fab fragment to each well.
18. Incubate 15 minutes at 37°C.
19. Wash plate 6 times with TBS/Tween.

20. Add 25□l BRL substrate to all wells.
21. Incubate 30 minutes at room temperature.
22. Add 25 □l BRL amplifier to all wells.
23. Watch the plate develop until the positive controls are strongly positive, and the negative controls remain clear. This usually takes 5-15 minutes.
24. Add 25 □l 0.3M H_2SO_4 to all wells to stop color development at desired time.
25. Read plates at 495nm.

1.6 Data analysis:

Take the mean of all ten negative control wells and determine the standard deviation of the negative controls. Add a value equal to three standard deviations to the mean of the negative control wells. Set this value as your cutoff in the assay. Any well with an OD value below the cutoff is scored as negative and anything with a value above the cutoff is scored as a putative positive. When a putative positive is detected, repeat the entire assay, beginning with a new PCR reaction. Any sample that scores above the cutoff in two independent PCRs is scored as a confirmed positive.

1.7 Quality Control

1. The positive control must turn positive and negative controls must turn negative for the assay to be valid.
2. Repeat every positive sample to confirm to positivity.

1.8 References

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